

PRECLINICAL RESEARCH

Plasma Level of Oxidized Low-Density Lipoprotein Is an Independent Determinant of Coronary Macrovasomotor and Microvasomotor Responses Induced by Bradykinin

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OBJECTIVES	We examined the relationship between coronary endothelium-dependent vasodilation in response to bradykinin (BK) and plasma levels of oxidized low-density lipoprotein (oxLDL) in subjects with normal coronary arteries.
BACKGROUND	It is unclear whether the plasma oxLDL level is a determinant of coronary endothelial function. Bradykinin plays an important role in regulating resting coronary tone and flow-mediated coronary vasomotion.
METHODS	Coronary blood flow (CBF) in the left anterior descending (LAD) coronary artery was assessed by quantitative angiography and a Doppler flow wire in 94 consecutive subjects with normal coronary arteries. The plasma oxLDL level was measured by enzyme-linked immunosorbent assay using DLH3R, a specific antibody against oxLDL.
RESULTS	Plasma levels of oxLDL in diabetic subjects ($n = 13$) were higher than those in non-diabetic subjects ($n = 81$). Plasma levels of oxLDL correlated with body mass index (BMI). Bradykinin at doses of 0.2, 0.6, and 2.0 $\mu\text{g}/\text{min}$ caused dose-dependent increases in diameter and CBF in the LAD coronary artery. By a univariate analysis, oxLDL levels significantly correlated with epicardial ($r = -0.30$, $p < 0.0001$) and resistant ($r = -0.36$, $p = 0.003$) coronary vasodilator responses to BK at 2.0 $\mu\text{g}/\text{min}$, whereas total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides were not associated with these coronary responses. In a stepwise multivariate analysis, oxLDL levels were significantly correlated with epicardial and resistant coronary vasomotor responses to BK, independent of age, gender, smoking status, other lipid levels, BMI, hypertension, and diabetes.
CONCLUSIONS	The plasma level of oxLDL is an appropriate surrogate for assessing coronary endothelial-dependent vasomotor function as estimated by responses to BK compared with conventional risk factors for atherosclerosis. (J Am Coll Cardiol 2004;44:451-7) © 2004 by the American College of Cardiology Foundation

Coronary endothelial vasomotor function is frequently impaired in the presence of coronary risk factors such as hypercholesterolemia, hypertension, diabetes mellitus, and smoking (1-3). Several lipid parameters affect coronary endothelial function (2,4,5). However, low-density lipoprotein (LDL) cholesterol has not always been shown to have an effect in multivariate analysis. Oxidized low-density lipoprotein (oxLDL) may be an important modulator of nitric oxide (NO) bioavailability and is a hallmark of atherogenesis (6,7). Early in vitro studies indicated that oxLDL cholesterol, but not LDL cholesterol, contributes to endothelial dysfunction by enhancing the degradation and inhibiting the synthesis of NO (8-10). Cholesterol-lowering therapy improves coronary or forearm endothelial function (11,12).

There is accumulating evidence that coronary endothelial

function is associated with future cardiovascular events and the progression of coronary artery disease (CAD) (13,14). It has been shown that plasma levels of oxLDL were elevated in patients with acute coronary syndrome and stable CAD (15,16). Nevertheless, the clinical importance of circulating oxLDL levels on coronary endothelial function has been poorly analyzed.

Bradykinin (BK) plays an important role in regulating resting coronary tone and flow-mediated coronary vasomotion (17). Bradykinin causes endothelium-dependent coronary vasodilation through the production of NO, prostacyclin, and endothelium-dependent hyperpolarizing factor (18). Recently, we reported that BK stimulates the release of tissue plasminogen activator as well as NO in human coronary circulation (19). At present, there are no data available regarding circulating parameters that may be responsible for BK-induced vasomotor responses in the coronary macrocirculation and microcirculation.

Thus, the present study was performed to examine whether the plasma oxLDL level is a significant and

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Abbreviations and Acronyms

BK	= bradykinin
BMI	= body mass index
CAD	= coronary artery disease
CBF	= coronary blood flow
HDL	= high-density lipoprotein
LAD	= left anterior descending
LDL	= low-density lipoprotein
NO	= nitric oxide
oxLDL	= oxidized low-density lipoprotein

independent determinant of BK-induced vasomotor responses of coronary conduit and resistance vessels compared with traditional coronary risk factors.

METHODS

Study patients. The study population consisted of 94 consecutive patients undergoing routine diagnostic catheterization and an assessment of coronary endothelial function for the evaluation of chest pain or myocardial ischemia on an electrocardiogram.

The study protocols were approved by the Ethical Committee on Human Research of our institution, and written informed consent was obtained from all of the patients. Patients with myocardial infarction, congestive heart failure, cardiomyopathy, valvular heart disease, coronary vasospastic angina, or organic stenosis (more than 25%) of coronary arteries were excluded from the study.

Smokers refrained from smoking for at least seven days before the present study to rule out the direct effects of oxidants contained in cigarette smoke. All vasoactive medications, including calcium channel blockers, nitrates, α -blockers, and angiotensin-converting enzyme inhibitors, as well as antioxidants, including statin and probucol, were discontinued for at least 48 h before the study. Smokers included subjects with a current history of tobacco use for more than one year. Subjects were considered to have hypertension if they had a blood pressure level of 140/90 mm Hg or more or if they were being treated. Subjects were considered to be diabetic if they had a fasting blood glucose level of 140 mg/dl or more or if they were already being treated. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m^2).

Protocol. Right and left cardiac catheterization was performed between 9 AM and 10 AM in the fasting state. After a diagnostic coronary angiographic study, a 0.014-inch Doppler-tipped guide wire (Jometrics FloWire; JoMed Inc., Rancho Cordova, California) was advanced through the Judkins catheter, as previously reported (19,20). The wire tip was positioned at the area between the proximal and middle segments of the left anterior descending (LAD) coronary artery that was free of any major branches within 1 cm from the tip, which permitted the measurement of an adequate blood flow velocity. All drugs were infused directly into the left main coronary artery via a 3-F coronary-infusion catheter (Cordis Endovas-

cular Systems Inc., Miami, Florida) at infusion rates ranging between 0.5 and 1 ml/min.

Baseline coronary angiography and measurement of coronary blood flow (CBF) velocity were performed, and subsequently, BK-induced vasodilation was estimated by dose-response curves obtained with incremental 2-min intracoronary infusions of BK. Coronary angiography and measurement of CBF velocity were performed after each infusion. Bradykinin was started at 0.2 $\mu\text{g}/\text{min}$, and then increased to 0.6 and 2.0 $\mu\text{g}/\text{min}$. After completing the protocol with the intracoronary injection of BK, we waited for at least 10 min, by which time the coronary diameter and CBF velocity had returned to the baseline values. Finally, nitroglycerin was given at 250 μg into the left coronary artery over 20 s. Maximal epicardial coronary vasodilation was measured after the infusion of nitroglycerin. Coronary angiography was performed at 2 min after the infusion of nitroglycerin. During the study, phasic and mean arterial pressures, heart rate, and 12-lead electrocardiograms were continuously monitored using a polygraph system (Nihon-Kohden Kogyo Co., Tokyo, Japan).

Quantitative coronary angiography and measurement of CBF. Coronary cineangiograms were recorded using a Philips cineangiographic system (Philips Medical Systems, Tokyo, Japan). The change in diameter of the LAD coronary artery was measured in a vessel segment 5 mm beyond the tip of the Doppler wire. Coronary angiograms were taken in the right caudal anterior oblique or right cranial anterior oblique position with adequate angulation to allow for clear visualization of the left coronary artery. Coronary angiograms were analyzed by quantitative coronary angiography using a Cardiovascular Measurement System (QCA-CMS; MEDIS Medical Imaging Systems, Leiden, The Netherlands). Measurements were made three times, and the average value was used for analysis. Peak CBF velocity was continuously monitored using a fast Fourier transform-based spectral analyzer (FloMap, Cardiometrics Inc., Mountain View, California). Coronary blood flow was calculated as $\pi \times \text{average peak CBF velocity} \times 0.125 \times (\text{arterial diameter})^2$. Vessel diameter and CBF velocity were analyzed by investigators blinded to the sequence of interventions and the laboratory data.

Preparation of BK. Bradykinin (Sigma Chemical Co., Tokyo, Japan) was diluted with physiologic saline at a concentration of 2 $\mu\text{g}/\text{ml}$ and sterilized at the Pharmacy Department, Shiga University of Medical Science Hospital.

Assay procedure for oxLDL and other lipids. Blood for measuring the plasma level of oxLDL was placed into a plain tube and centrifuged at 3,000 rpm for 15 min at 4°C. The resulting plasma was stored at 4°C until the assay. Plasma oxLDL levels were measured using a specific immunometric assay for human oxLDL using an enzyme-linked immunosorbent assay kit (Kyowa Medex Co., Tokyo, Japan) (21), which used a modification of a method previously reported. Briefly, this assay system uses two antibodies against human oxLDL, one a monoclonal antibody against oxidized phosphatidylcholine (22) and the other a polyclonal antibody against human

Table 1. Clinical Characteristics

Age (yrs)	58 ± 1 (33-79)
Gender (male/female)	68/26
Smoking status (%)	42 (45%)
Hypertension (%)	30 (32%)
Diabetes mellitus (%)	13 (14%)
Body mass index (kg/m ²)	23.5 ± 3.2 (16.7-34.0)
Total cholesterol (mg/dl)	188 ± 4 (112-271)
Triglycerides (mg/dl)	126 ± 6 (30-343)
HDL cholesterol (mg/dl)	46 ± 1 (21-105)
LDL cholesterol (mg/dl)	121 ± 3 (49-195)
Oxidized LDL (U/ml)	13.8 ± 0.9 (2.4-52.5)

Data are presented as mean ± SE (range) or number (%) of subjects.
HDL = high-density lipoprotein; LDL = low-density lipoprotein.

apolipoprotein B, and measures oxLDL by sandwiching it between the two antibodies. Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides and LDL cholesterol were measured using enzymatic assays.

Statistical analysis. Data are expressed as the mean value ± SEM. Unpaired Student *t* test was used for comparison of continuous variables. Univariate and stepwise multivariate linear regression analyses were used to detect independent predictors of epicardial and resistant coronary vasodilation induced by BK (StatView version 5.0). A value of *p* < 0.05 was considered statistically significant.

RESULTS

Subject characteristics. The baseline characteristics of the study group are shown in Table 1. The study population undergoing coronary angiography was predominantly male and older, and had a combination of risk factors that required cardiac medication.

Plasma levels of oxLDL. Plasma levels of oxLDL in diabetic subjects were higher (21.6 ± 0.7 IU/ml, range 8.2 to 52.5 IU/ml) than those in non-diabetic subjects (12.9 ± 3.6 IU/ml, range 2.4 to 44.6 IU/ml, *p* = 0.0002) (Fig. 1, left). Plasma levels of oxLDL correlated with BMI (*r* =

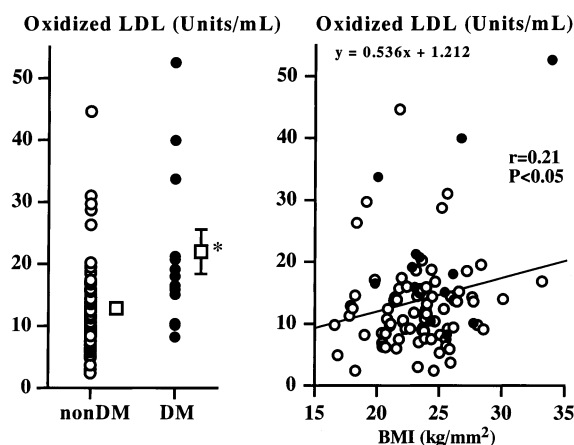


Figure 1. Effects of diabetes mellitus (DM) and body mass index (BMI) on plasma levels of oxidized low-density lipoprotein (LDL). **p* = 0.0002 versus the non-diabetic subjects. Values represent mean ± SEM. Open circles represent non-diabetic subjects; closed circles represent diabetic subjects.

Table 2. Correlations Between Plasma Oxidized LDL Level and Other Parameters

	<i>r</i>	<i>p</i> Value
Age (yrs)	0.111	0.2857
Gender (male = 1)	-0.15	0.1477
Smoking (yes = 1)	0.085	0.4147
Diabetes mellitus (yes = 1)	0.377	0.0002
Hypertension (yes = 1)	0.091	0.3825
BMI (kg/m ²)	0.205	0.0475
Total cholesterol (mg/dl)	0.125	0.2314
Triglycerides (mg/dl)	-0.09	0.3864
HDL cholesterol (mg/dl)	-0.071	0.4986
LDL cholesterol (mg/dl)	0.182	0.0797

BMI = body mass index; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

0.21, *p* < 0.05) (Fig. 1, right; Table 2). However, there was no significant difference between diabetic and non-diabetic subjects with regard to BMI (24.8 ± 1.0 vs. 23.3 ± 0.3 kg/m²). As shown in Table 2, plasma levels of oxLDL did not correlate with levels of total cholesterol, LDL cholesterol, HDL cholesterol, or triglycerides, and were independent of age, gender, smoking status, and hypertension (*n* = 94, respectively).

Coronary vasomotor responses to BK and plasma levels of oxidized LDL. Intracoronary infusion of BK (0.2, 0.6, and 2.0 μg/min) caused dose-dependent increases in epicardial coronary diameter and CBF (Table 3) without significantly changing the arterial pressure or heart rate in any of the patients. The mean value of epicardial coronary vasodilation induced by nitroglycerin (250 μg) was 23.0 ± 1.7% in all of the patients.

In simple linear regression analyses, increases in epicardial coronary diameter and CBF in response to BK (0.2, 0.6, and 2.0 μg/min) showed significant negative correlations with plasma levels of oxLDL, respectively (*n* = 94) (Table 3). As shown in Figure 2, increases in epicardial coronary diameter and CBF in response to BK (2.0 μg/min) showed significant negative correlations with plasma levels of oxLDL. As shown in Figure 3, increases in epicardial coronary diameter and CBF in response to BK (2.0 μg/min) were not correlated with plasma levels of LDL cholesterol. The epicardial coronary vasodilation induced by nitroglycerin

Table 3. Correlations Between Plasma oxLDL Levels and Coronary Vasomotor Responses to Bradykinin

	Mean ± SE	<i>r</i>	<i>p</i> Value
Increases in epicardial coronary diameter (%)			
BK (0.2 μg/min)	10.4 ± 0.9	-0.253	0.0137
BK (0.6 μg/min)	15.9 ± 1.2	-0.308	0.0025
BK (2.0 μg/min)	21.8 ± 1.4	-0.352	0.0005
Increases in coronary blood flow (%)			
BK (0.2 μg/min)	87.5 ± 6.8	-0.262	0.0108
BK (0.6 μg/min)	162.5 ± 10.1	-0.362	0.0003
BK (2.0 μg/min)	258.9 ± 12.3	-0.452	<0.0001

Data are presented as mean ± SE.

BK = bradykinin; ox-LDL = oxidized low-density lipoprotein.

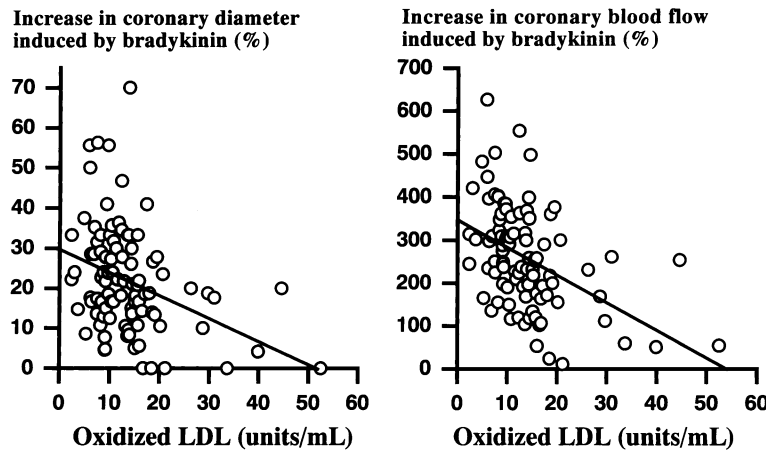


Figure 2. Relationship between plasma oxidized low-density lipoprotein (LDL) levels and coronary vasomotor responses to bradykinin (2.0 $\mu\text{g}/\text{min}$).

(250 μg) was not correlated with the plasma level of oxLDL ($p = 0.07$, $n = 94$).

As shown in Table 4, epicardial coronary vasodilation induced by BK (2.0 $\mu\text{g}/\text{min}$) was associated with diabetes mellitus and BMI but was not correlated with age, gender, smoking status, or hypertension ($n = 94$, respectively). The epicardial coronary vasodilation induced by BK (2.0 $\mu\text{g}/\text{min}$) was not correlated with plasma levels of cholesterol, LDL cholesterol, HDL cholesterol, or triglycerides ($n = 94$, respectively). In a stepwise multivariate analysis, the plasma oxLDL level and BMI were significant independent determinants of the epicardial coronary vasodilation induced by BK (2.0 $\mu\text{g}/\text{min}$) (Table 4).

As shown in Table 5, the increases in CBF induced by BK (2.0 $\mu\text{g}/\text{min}$) were associated with hypertension, diabetes mellitus, and BMI but were not correlated with age, gender, or smoking status ($n = 94$, respectively). The increases in CBF induced by BK (2.0 $\mu\text{g}/\text{min}$) were also not correlated with plasma levels of cholesterol, LDL cholesterol, HDL cholesterol, or triglycerides ($n = 94$, respectively). In a stepwise multivariate analysis, the plasma oxLDL level and hypertension were significant independent

determinants of the increases in CBF induced by BK (2.0 $\mu\text{g}/\text{min}$) (Table 5).

DISCUSSION

The present study is the first to demonstrate that the plasma level of oxLDL, but not conventional lipid parameters, is significantly correlated with coronary macrovasomotor and microvasomotor responses induced by BK. Thus, the plasma level of oxLDL may provide useful information for predicting endothelial dysfunction underlying the progression of CAD and future cardiovascular events compared with conventional lipid parameters.

Plasma level of oxLDL. In the present study, plasma levels of oxLDL in diabetic patients were higher than those in non-diabetic patients. Factors that may promote LDL oxidation in diabetic patients include antioxidant deficiencies, increased production of reactive oxygen species, and protein glycation. The plasma oxLDL level correlated with BMI but not with conventional lipid parameters, age, gender, or smoking status. Obesity often coexists with diabetes, but BMI did not differ between diabetic and

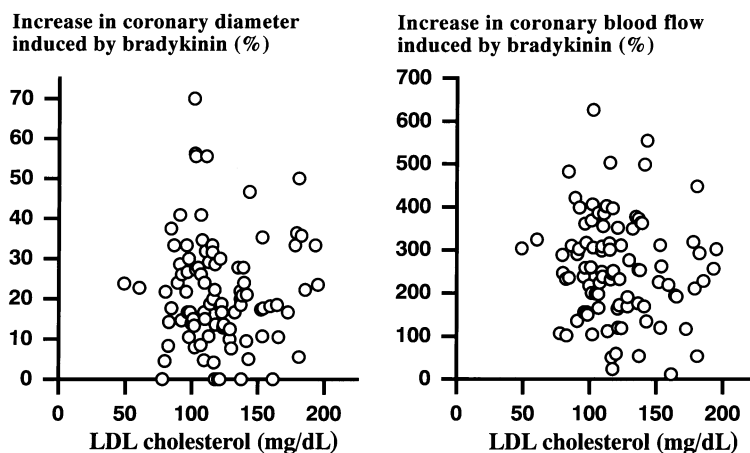


Figure 3. Relationship between plasma low-density lipoprotein (LDL) cholesterol levels and coronary vasomotor responses to bradykinin (2.0 $\mu\text{g}/\text{min}$).

Table 4. Univariate and Multivariate Linear Models of Epicardial Coronary Vasomotor Responses to Bradykinin

Variables	Univariate Correlation Coefficient	p Value	Multivariate Beta-Coefficient (SE)	p Value
Age (yrs)	−0.066	0.5281		
Gender (male = 1)	0.117	0.2599		
Smoking (yes = 1)	0.1	0.3392		
Diabetes mellitus (yes = 1)	−0.283	0.0058		
Hypertension (yes = 1)	−0.178	0.0864		
BMI (kg/m ²)	−0.327	0.0013	−1.130 (0.409)	0.007
Total cholesterol (mg/dl)	0.078	0.4567		
Triglycerides (mg/dl)	0.12	0.2499		
HDL cholesterol (mg/dl)	0.0069	0.5069		
LDL cholesterol (mg/dl)	−0.008	0.9426		
Oxidized LDL (U/ml)	−0.352	0.0005	−0.484 (0.156)	0.0026

Abbreviations as in Table 2.

non-diabetic patients. Keaney *et al.* (23) recently reported that obesity, as measured by BMI, is independently associated with oxidative stress in the Framingham Heart Study.

The clinical importance of circulating oxLDL has not been established, although oxLDL is involved in atherogenesis by inducing smooth muscle cell proliferation and smooth muscle foam cell generation (6,7). Recent reports have shown that the plasma level of oxLDL was associated with the severity of acute coronary syndromes and CAD (15,16,24). Nevertheless, it is unclear whether plasma levels of oxLDL may be associated with coronary vasomotor reactivities in subjects with angiographically normal coronary arteries. Several methods are available for estimating oxidative stress, but there are few appropriate assays that are sensitive and specific for oxLDL. A monoclonal antibody, DLH3R (22), reacts with oxLDL but not with native, acetylated, or malondialdehyde-treated LDL. Using this probe, we reported that the plasma level of oxLDL is a useful marker of oxidative stress in patients with heart failure (25). Further studies are needed to elucidate the mechanisms underlying the increase in circulating oxLDL levels.

OxLDL level and coronary vasomotor response to BK.

In the present study, oxLDL levels but not other lipid parameters correlated inversely and significantly with epicardial and resistant coronary vasodilation induced by BK. Bradykinin regulates the resting tone and flow-mediated vasodilation in human coronary circulation (17). To investigate whether the oxLDL level is an independent determinant of coronary vasomotor responses to BK, a multivariate analysis was performed in which conventional coronary risk factors were entered as independent variables. We demonstrated that the oxLDL level, but not other lipid parameters, is an independent and powerful determinant of coronary macrovasomotor and microvasomotor responses to BK. Traditional coronary risk factors such as hypercholesterolemia, hypertension, diabetes mellitus, and cigarette smoking have been shown to contribute to endothelial dysfunction in coronary and peripheral vessels, and these effects are not limited to atherosclerotic sites (1–3). Nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate-mediated superoxide production may account for endothelial dysfunction and the increased risk for cardiovascular events in hypertensive patients (26). Non-insulin-dependent diabetes mellitus may cause endothelial dysfunction through hyperglycemia, hyperinsulinemia, and

Table 5. Univariate and Multivariate Linear Models of Resistance Coronary Vasomotor Responses to Bradykinin

Variables	Univariate Correlation Coefficient	p Value	Multivariate Beta-Coefficient (SE)	p Value
Age (yrs)	−0.092	0.38		
Gender (male = 1)	−0.057	0.5879		
Smoking (yes = 1)	−0.048	0.6445		
Diabetes mellitus (yes = 1)	−0.284	0.0055		
Hypertension (yes = 1)	−0.246	0.0166	−52.678 (23.247)	0.0258
BMI (kg/m ²)	−0.263	0.0105		
Total cholesterol (mg/dl)	−0.061	0.5584		
Triglycerides (mg/dl)	0.048	0.6461		
HDL cholesterol (mg/dl)	0.026	0.8006		
LDL cholesterol (mg/dl)	−0.103	0.3223		
Oxidized LDL (U/ml)	−0.452	<0.0001	−6.166 (1.302)	<0.0001

Abbreviations as in Table 2.

lipid disorders (27). Our results showed that hypertension causes coronary microvascular responses to BK and that diabetes mellitus is related to coronary macrovascular or microvascular responses to BK.

The present study showed that BMI had an independent effect on coronary macrovascular responses to BK even after a multivariate analysis of conventional coronary risk factors. It has recently been shown that BMI is an important determinant of CBF responses to acetylcholine in patients with normal or mildly diseased coronary arteries (28) and of forearm blood flow responses to BK in healthy subjects (29). Brachial artery vasodilation induced by acetylcholine is blunted in obese subjects, and this attenuated response is reversed by vitamin C, suggesting that oxidative stress may contribute to endothelial dysfunction in human obesity (30).

Long-term cigarette smoking impairs endothelium-dependent coronary and forearm vasodilation via increased oxidant stress (31,32). Thus, it is possible that coronary responses to BK and the plasma level of oxLDL may have been normalized by the cessation of smoking before the present study.

Early studies showed that the level of total cholesterol was inversely related to endothelium-dependent vasodilation, regardless of the presence or absence of CAD (1,2). On the other hand, *in vitro* animal experiments showed that an increase in oxLDL, but not native LDL cholesterol, causes endothelial dysfunction by inhibiting the synthesis and enhancing the degradation of NO (8-10). It has been shown that LDL size, triglycerides, and HDL cholesterol contribute to endothelium-dependent vasomotor responses (33). In the present study, the ranges of conventional lipid parameters may have been relatively narrow.

Recent studies have shown that abnormal coronary vasoreactivity can predict the progression of atherosclerotic disease and increased cardiovascular risk in patients at risk for CAD (13,14). Heitzer *et al.* (34) demonstrated that increased vascular oxidative stress may play an important role in mechanisms for endothelial dysfunction and in the pathogenesis of cardiovascular events. Therefore, the plasma level of oxLDL could be a more useful predictor of cardiovascular events than conventional coronary risk factors.

Study limitations. Some patients received medications such as statins, probucol, calcium antagonists, angiotensin-converting enzyme inhibitors, or angiotensin-1 receptor antagonists before the study. These agents have been shown to have beneficial effects on endothelial function, but the present study failed to address the beneficial effects of these drugs (data not shown). Thus, we must further examine the effects of pharmacologic intervention on the plasma level of oxLDL and endothelial function.

Antibodies against oxLDL recognize different epitopes of complex structures formed during the oxidation of lipoproteins. The accurate quantitation of oxLDL will be achieved when the epitopes are better delineated.

In conclusion, the present study showed that the plasma

level of oxLDL is an independent determinant of endothelial function in coronary macrocirculation and microcirculation in comparison with conventional lipid parameters. Based on the concept that endothelial dysfunction precedes cardiovascular events, measurement of the plasma oxLDL level may provide useful information for predicting future cardiovascular events.

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